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Peroxisome Proliferation due to Di(2-ethylhexyl) Phthalate (DEHP): Species Differences and Possible Mechanisms*

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The exposure of cultured rat hepatocytes to mono(2-ethylhexyl)phthalate (MEHP) for 72 hr resulted in marked induction of peroxisomal enzyme activity (β-oxidation; cyanide-insensitive palmitoyl CoA oxidase) and concomitant increases in the number of peroxisomes. Similar treatment of cultured guinea pig, marmoset, or human hepatocytes revealed little or no effect of MEHP. In order to eliminate possible confounding influences of biotransformation, the proximate peroxisome proliferator(s) derived from MEHP have been identified. Using cultured hepatocytes these agents were found to be metabolite VI [mono(2-ethyl-5-oxohexyl) phthalate] and metabolite IX [mono(2-ethyl-5-hydroxyhexyl) phthalate]. The addition of these "active" metabolites to cultured guinea pig, marmoset, or human hepatocytes again revealed little effect upon peroxisomes or related enzyme activities (peroxisomal β-oxidation or microsomal lauric acid hydroxylation). These studies demonstrate a marked species difference in the response of hepatocytes to MEHP-elicited peroxisome proliferation. Preliminary studies have also suggested that peroxisome proliferation due to MEHP may be due to an initial biochemical lesion of fatty acid metabolism.

Introduction

Perhaps one of the most striking toxicological effects of di(2-ethylhexyl) phthalate (DEHP) and related phthalate esters is their ability to elicit hepatomegaly in rodents (*1,2*). This phenomenon is explained, in part, by an increase in the cytoplasmic volume of the hepatocytes due to a marked proliferation of peroxisomes and smooth endoplasmic reticulum (*2,3*). The increased volume density of these organelles is accompanied by induction of peroxisomal β-oxidation and microsomal cytochrome P-452-mediated fatty acid hydroxylation (*4–6*). These properties are shared with many hypolipidemic drugs (e.g., clofibrate, fenofibrate, ciprofibrate) and a variety of other chemicals such as phenoxyacetic acid herbicides (*7–14*).

Several hypolipidemic drugs have elicited hepatocellular carcinoma in rodents when administered for long periods of time at relatively high dose levels (*9,15–22*). On this basis, Reddy and co-workers (*23*) have proposed that peroxisome proliferators represent a novel class of chemical carcinogen, which in general are nonmutagenic and do not interact covalently with DNA (*18,19,24–26*). It would appear that DEHP belongs to this class of chemical, since the majority of evidence suggests it to lack significant direct genotoxic potential (*27–29*). However, dietary concentrations of DEHP of up to 12,000 ppm have increased the incidence of hepatocellular carcinoma in rats and mice in a two-year chronic toxicity study (*30*).

It has been postulated (*16*) that the carcinogenic potential of peroxisome proliferators arises from "active" O_2 genotoxicity, caused by imbalanced increases in H_2O_2-generating oxidases (e.g., acyl CoA oxidase) and H_2O_2-degrading enzymes (catalase).

Some studies have reported marked species difference in response to peroxisome proliferators. For example, several agents, which are active in rats, have failed to elicit peroxisome proliferation in dogs, marmosets, rhesus monkeys, or guinea pigs (*4,5,31–35*). However, ciprofibrate, a very potent hypolipidemic drug, has been reported to cause hepatic peroxisome proliferation in both rhesus and cynomolgus monkeys (*36*). Examination of human liver biopsy material, obtained from patients receiving clofibrate or fenofibrate, has demonstrated marginal or no increases in peroxisome numerical or volume densities (*32,37–41*).

In vivo, studies of species differences in response to peroxisome proliferators are frequently compromised by variations in administered dose, target organ dose, or differences in routes and rates of biotransformation. For example, guinea pigs produce only small quantities of oxidized DEHP metabolites (*42*), while the marmoset appears to absorb only a small proportion of an orally-administered dose of DEHP (*43*).

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In these studies we have attempted to eliminate such confounding factors by identifying the proximate proliferator(s) derived from DEHP and examining the species variation in response utilizing the active metabolites. In addition, we also report data suggesting a possible mechanism for DEHP elicited peroxisome proliferation in rats.

Materials and Methods

Materials

Mono(2-ethylhexyl) phthalate (MEHP), and di(2-ethylhexyl) phthalate (DEHP) were supplied by Petrochemicals and Plastics Division, ICI PLC (Wilton, Middlesborough, Cleveland, UK). 1-^{14}C-lauric acid and 1-^{14}C-palmitic acid were obtained from Amersham International PLC (Amersham, UK).

Leibowitz L15 culture medium, fetal bovine serum, tryptose phosphate broth and collagenase were obtained from Flow Laboratories (Irvine, UK). All other chemicals were of the highest available purity and were obtained from Sigma Chemical Company (Poole, UK) or BDH (Liverpool, UK).

Animals

Experiments were performed on male Alderley Park rats (Wistar-derived, 180–220 g), male Alderley Park guinea pigs (Duncan and Hartley, 400–500 g) and male marmosets (*Callithrix jacchus*, 350–500 g). All animals were obtained from the Animal Breeding Unit, Imperial Chemical Industries, Pharmaceuticals Division (Alderley Park, Cheshire, UK). Rats and guinea pigs were allowed food *ad libitum*, while marmosets were fed once daily. All animals had free access to water. A 12-hr light/dark cycle (0600–1800) was operated. Prior to isolation of hepatocytes rats and guinea pigs were killed by inhalation of excessive diethyl ether, and marmosets were killed by injection of a lethal dose of pentobarbital.

Hepatocyte Isolation and Culture

Rat and guinea pig hepatocytes were isolated by a two-step *in situ* perfusion technique as described previously (*44*). Strict aseptic techniques were observed throughout the procedure. Marmoset hepatocytes were isolated in a similar manner to rat hepatocytes, except the perfusion media were: 500 mL PBS (200 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4; 20 mM $Na_2HPO_42H_2O$) followed by 500 mL of PBS/EGTA (PBS containing 0.5 mM EGTA) and finally 200 mL (with recirculation) of Hanks BSS (200 mM NaCl; 5.3 mM KCl; 0.4 mM Na_2HPO_4; 0.4 mM KH_2PO_4; 5.5 mM glucose) containing dispase (300 mg), collagenase (100 mg), hyaluronidase (100 mg), deoxyribonuclease (10 mg) and $CaCl_22H_2O$ (2.8 mM), pH 7.4. After about 20 min of perfusion with enzyme-containing solution, the liver was removed and hepatocytes collected as described for rat liver previously.

Human liver was obtained from brain-dead renal transplant donors after compliance with ethical and legal requirements. Light and electron microscopic examination of the donor livers revealed normal morphological and ultrastructural characteristics. The outer capsule of the liver was removed and the liver sliced with a degreased microtome blade into slices of approximately 0.5 mm. Liver slices (10 g) were incubated in 25 mL PBS at 37°C for 10 min. The PBS was decanted, and two further washes in PBS and two washes in PBS/EGTA performed. Finally, 20 mL Hank's BSS containing dispase (60 mg), collagenase (20 mg), hyaluronidase (20 mg), deoxyribonuclease (2 mg) and $CaCl_2$ (2.8 mM) were added, and the slices incubated at 37°C for 30 min. The hepatocytes were collected by filtration and centrifugation as previously described.

The isolated cells were suspended in CL15 medium i.e., Leibowitz L15 medium containing fetal bovine serum (8.3%), tryptose phosphate broth (8.3%), penicillin G (41.3 IU/mL), streptomycin sulfate (8.2 μg/mL), glutamine (241 μg/mL), insulin (10^{-6} M) and hydrocortisone (10^{-5} M). Vitamin C (50 mg/L) was included in the guinea pig, marmoset, and human hepatocyte cultures. The viabilities of the hepatocyte preparations (> 95% for animals, > 80% for human) were determined by trypan blue dye exclusion. Falcon tissue culture flasks (25 cm^2) were seeded with 2×10^6 viable hepatocytes contained in 4 mL of CL15 medium. The flasks were incubated at 37°C in air. At 4, 24, 48, and 72 hr after seeding, the spent medium and any detached cells were aspirated and fresh medium applied. DEHP metabolites dissolved in dimethylformamide (DMF) were added to the monolayers at each 24-hr medium change. Additions of DMF never exceeded 10 μL per flask, and this concentration produced no noticeable cytotoxicity and had no effect upon the parameters measured. Evidence of cytotoxicity was manifested by blebbing, rounding up of cells, and detachment from the flasks.

Harvesting of Cell Monolayers

At 96 hr after seeding, the monolayer cultures were harvested. The medium was discarded and the monolayer washed in 2 mL of SET buffer (0.25 M sucrose/5 mM EDTA/20 mM Tris·HCl, pH 7.4). The cells were removed from the flask by scraping with a rubber policeman into 1 mL of SET buffer and were disrupted by sonication and stored at −70°C. The enzyme activities were stable for several weeks at this temperature.

Peroxisomal Enzyme Activity

Cyanide-insensitive fatty acid β-oxidation is a marker enzyme for peroxisomes (*45*). Peroxisomal β-oxidation was measured in cell sonicates as the palmitoyl-CoA dependent reduction of NAD^+ in the presence of cyanide (to inhibit the mitochondrial reoxidation of NADH) as described previously (*46*) with some modifications. The assay medium contained: 60 mM Tris·HCl pH 8.3, 50 μM FAD, 370 μM NAD^+, 94 mM nicotinamide, 2.8

mM dithiothreitol, 2mM KCN and 0.15 mg/mL bovine serum albumin (fatty acid free). The enzymatic activity was expressed as nmole NAD^+ reduced/min/mg protein.

Lauric Acid Hydroxylase Activity

Cytochrome P-450-mediated lauric acid hydroxylase activity (LAH) was assayed essentially as described before (*47*) with some modifications. Reaction mixtures (37°C) contained 1 to 2 mg cellular protein, 255 nmole (1.7 μCi) 1-^{14}C-lauric acid, 1.5 μmole NADPH in 2 mL of 66 mM Tris·HCl (pH 7.4). The reaction was terminated after 10 min by the addition of 500 μL of 1 M HCl and the ^{14}C-lauric acid and ^{14}C-hydroxylated products were extracted in 5 mL diethyl ether. A sample of the ether extract was evaporated to dryness under N_2 and redissolved in methanol. The products were separated by TLC on Whatman KC18F reversed-phase thin-layer chromatography plates developed in methanol:water:glacial acetic acid (80:19:5:0.5). The radioactive areas were localized by autoradiography, scraped from the plates, and the ^{14}C-radioactivity determined by liquid scintillation counting. LAH activity was expressed as nmole hydroxylated lauric acid formed/min/mg protein.

Protein Assay

Protein was determined by the method of Lowry et al. (*48*) by using bovine serum albumin standards.

Fatty Acid Oxidation by Isolated Hepatocytes

The production of $^{14}CO_2$ and ^{14}C-acid-soluble material from 1-^{14}C-palmitic acid was determined in isolated rat hepatocytes by the method of Christiansen et al. (*49*).

Fatty Acid Oxidation by Isolated Mitochondria

Rat liver was homogenized (Potter-Elvehjem glass/Teflon homogenizer, 1/20 in. clearance) in five volumes of ice-cold 0.3 M sucrose. The homogenate was centrifuged at 850 g_{av} for 10 min at 4°C. The resultant supernatant was centrifuged at 10,000 g_{av} for 20 min at 4°C and the resultant pellet resuspended in 0.3 M sucrose (1 mL/g liver). The functional integrity of the mitochondria was assessed by determination of the P/O ratio. Using pyruvate as substrate, preparations having a P/O ratio of less than 2.5 were discarded.

The metabolism of acyl-L-carnitines was studied in 2,4-dinitrophenol-uncoupled mitochondria in the presence of arsenate and malonate. In this situation O_2 consumption (measured polarographically using a Clarke type O_2 electrode) stoichiometrically reflects the β-oxidation of the acyl-L-carnitine. A fuller explanation of this system has been given by Sherratt and Osmundsen (*50*).

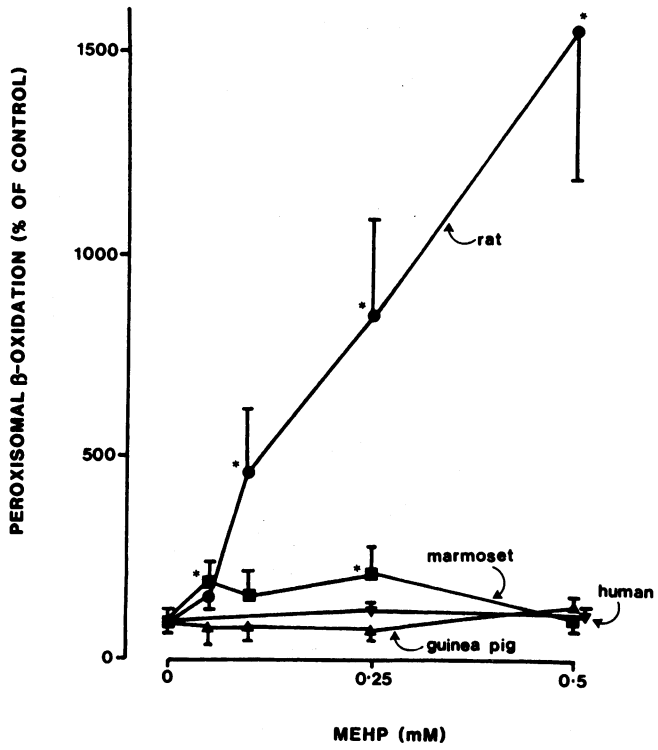


FIGURE 1. Effect of mono(2-ethylhexyl) phthalate (MEHP) on peroxisomal β-oxidation of cultured rat, guinea pig, marmoset, and human hepatocytes. Cell cultures were exposed to MEHP for 72 hr (see Methods). Values are mean ± SD (n = 3–8). Asterisks denote values significantly different from respective control, $p < 0.05$. Control values (nmole/min/mg protein) were: rat, 0.71 ± 0.18; guinea pig, 0.16 ± 0.08; marmoset, 0.85 ± 0.46; human, 0.11 ± 0.04.

Statistics

Statistical comparisons were carried out by using Student's t-test. A level of significance of $p < 0.05$ (two-tailed) was chosen.

Results

Species Differences in Peroxisome Proliferation due to MEHP and Its Metabolites

Since DEHP is hydrolyzed in the intestine to MEHP and is absorbed as such (*51,52*), the present *in vitro* investigations have utilized MEHP. The exposure of rat hepatocyte cultures to MEHP for 72 hr resulted in a marked (15-fold at 0.5 mM) increase in the activity of cyanide-insensitive palmitoyl CoA oxidation (PCO) (Fig. 1). This was paralleled by increases in the numerical and volume densities of peroxisomes (data not shown). Similar treatment of guinea pig and human hepatocytes resulted in no stimulation of PCO. Marmoset cultures occasionally showed very small increases in PCO, but these changes did not appear to be related to the concentration of MEHP (Fig. 1). The guinea pig,

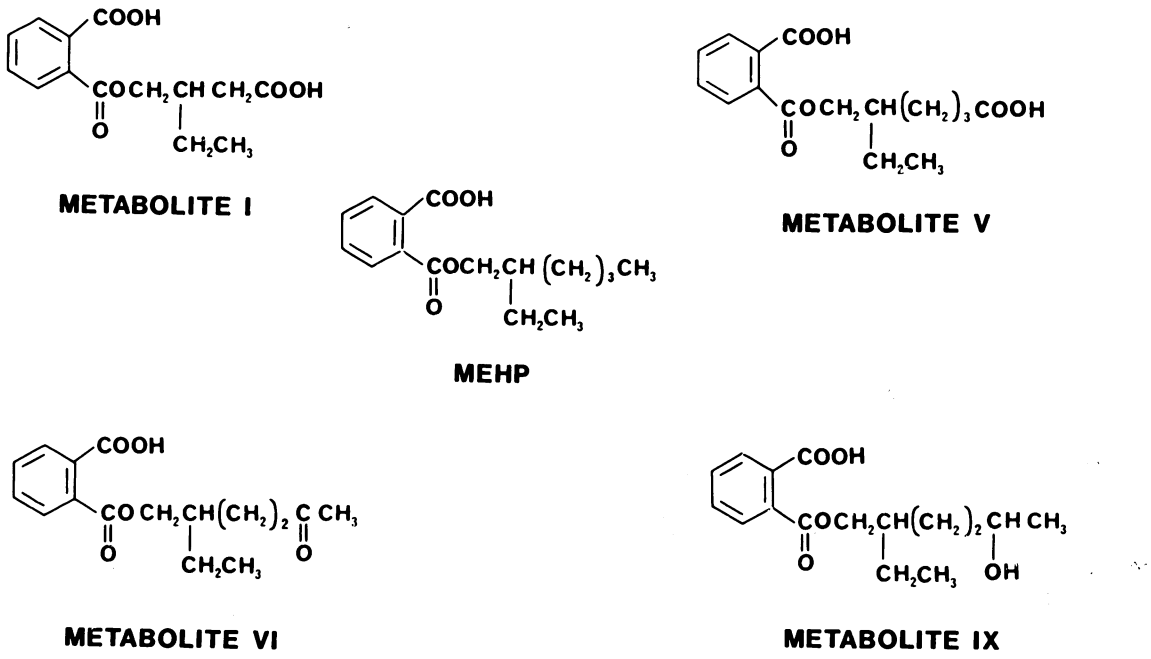


FIGURE 2. Structures of DEHP metabolites.

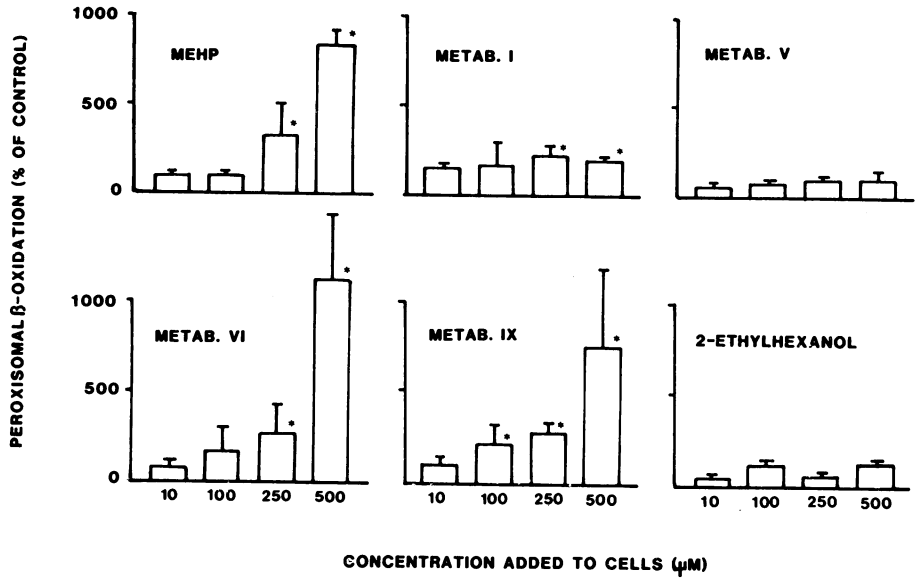
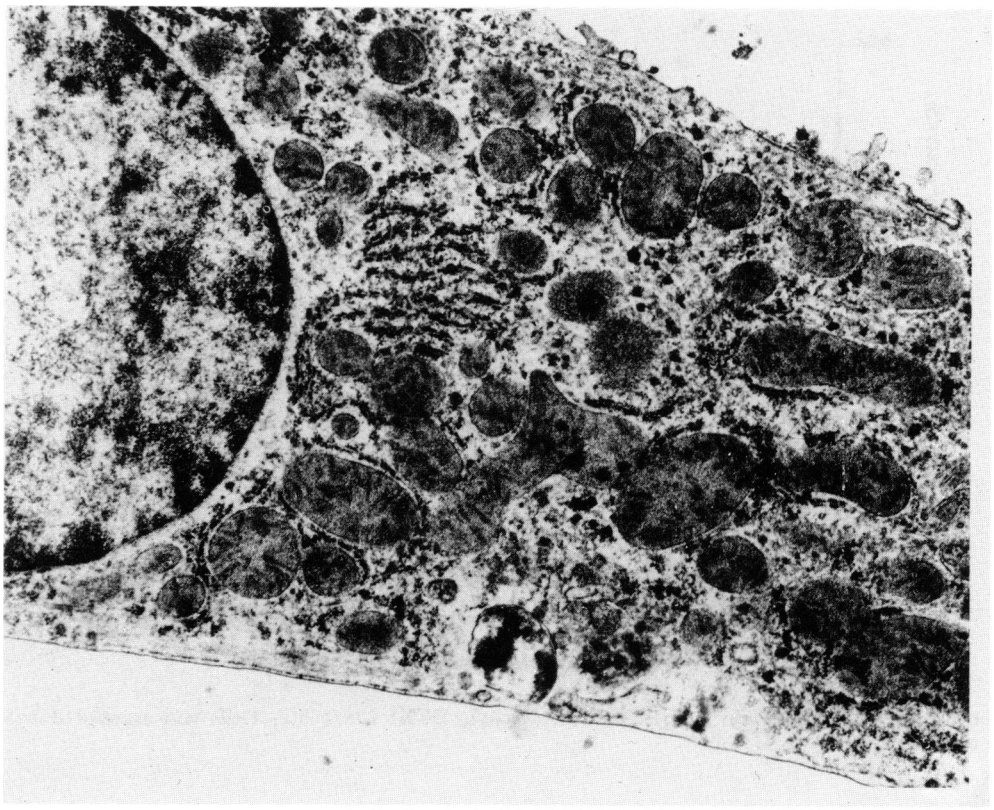


FIGURE 3. Induction of peroxisomal β-oxidation by DEHP metabolites in cultured rat hepatocytes. Cultures were exposed to various (0–0.5 mM) concentrations of DEHP metabolites for 72 hr. Values are means ± SD (n = 4–6); asterisks denote values significantly different from control, $p < 0.05$.

marmoset, and human cultures were considered to be of high viability and metabolically competent since phenobarbitone was able to induce cytochrome P-450-mediated ethoxycoumarin-*O*-deethylase in parallel cultures (Elcombe, unpublished data).

The use of MEHP in cultured cells, although eliminating differences in the hydrolysis of DEHP to MEHP and differences in the bioavailability of MEHP to the liver, does not allow us to eliminate possible species differences in biotransformation of MEHP. Hence metabolites of MEHP were isolated from rat urine (*53*) and hepatocytes from rat, guinea pig, marmoset, and human liver exposed to each metabolite for 72 hr. The systematic names and structures of the metabolites utilized are indicated in Figure 2. Following the exposure of rat cells to the ω-oxidation products of MEHP (metabolites I and V) or 2-ethylhexanol, little alteration in PCO was observed (Fig. 3). However, marked stimulation of PCO



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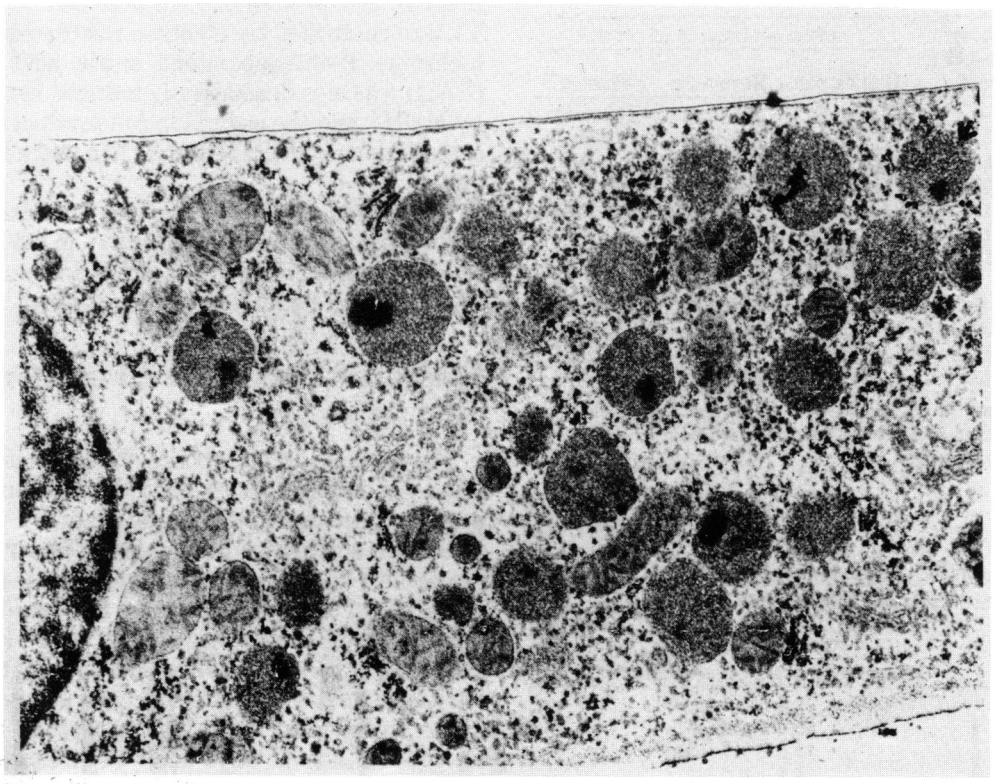

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FIGURE 4. Electron photomicrographs of cultured rat hepatocytes: (a) control hepatocyte, (b) metabolite VI-exposed rat hepatocyte, (p) represents a peroxisome. × 24,000.

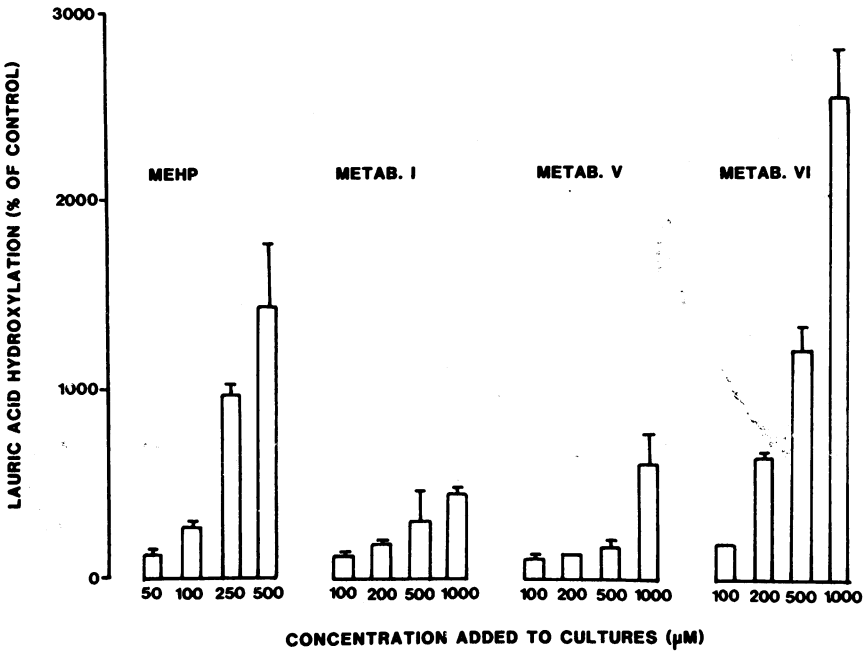

FIGURE 5. Induction of lauric acid hydroxylase (LAH) in rat hepatocytes by DEHP metabolites. Cells were exposed to the various metabolites for 72 hr. Values are means ± SD (n = 4–6).

Table 1. Effect of metabolite VI on palmitoyl CoA oxidation (PCO) and lauric acid hydroxylation (LAH) in cultured guinea pig, marmoset, and human hepatocytes.[a]

Enzyme	Concn Metab. VI, mM	Enzyme activity, % of control		
		Guinea pig	Marmoset	Human
PCO	0	100 ± 5	100 ± 54	100 ± 40
	0.1	ND	202 ± 18	ND
	0.25	166 ± 32	ND	ND
	0.5	118 ± 1	86 ± 64	73 ± 12
	1.0	139 ± 29	202 ± 18	104 ± 24
	1.5	139 ± 28	ND	ND
	2.0	143 ± 34	ND	69 ± 17
LAH	0	ND	100 ± 7	100 ± 6
	0.5	ND	95 ± 8	77 ± 15
	1.0	ND	147 ± 14	67 ± 14
	2.0	ND	102 ± 8	46 ± 7

[a] Cells were exposed to metabolite VI for 72 hrs (see Methods). Values are expressed as % of respective control (mean ± SD, n = 3–8); ND = not determined.

Table 2. Inhibition of 1-^{14}C-palmitic acid oxidation by metabolite VI in isolated rat hepatocytes.

Concn, VI, mM	^{14}C-Palmitate oxidation, nmole oxidized/10^6 cells/30 min[a]
0	20.1 ± 1.1 (100)
0.01	19.4 ± 0.2 (96)
0.10	14.6 ± 1.1 (72)
0.50	14.4 ± 0.7 (71)

[a] Values are mean ± SD (n = 4) and represent the total of $^{14}CO_2$ released and acid-soluble ^{14}C (acetate, hydroxybutyrate, and acetoacetate). Values in parentheses are % of control (in the absence of VI).

was seen after exposure of the rat cultures to metabolites VI and IX (the ω-1 oxidation products of MEHP) (Fig. 3). The proliferation of peroxisomes by metabolite VI was confirmed by electron microscopy (Fig. 4). Cytochrome P-452-dependent lauric acid hydroxylation (LAH) was also dramatically induced in rat hepatocytes by MEHP and the ω-1 oxidation product (VI), while the ω-oxidation products (I and V) had much less effect (Fig. 5).

In marked contrast, the ω-1 oxidation product of MEHP (i.e., metabolite VI, active in rat cells) had little or no effects upon PCO or LAH in marmoset, guinea pig or human hepatocytes (Table 1).

Effects of Metabolite VI on Hepatic Fatty Acid Oxidation

The addition of metabolite VI to a suspension of isolated rat hepatocytes resulted in a concentration-dependent decrease in the oxidation of 1-^{14}C-palmitic acid (Table 2), a decrease of 30% being observed at 0.1 mM metabolite VI. It should be noted that the experimental design (i.e., using 1-^{14}C-labeled fatty acid) only enabled the effects of metabolite VI on long chain fatty acid metabolism to be determined. The major proportion of fatty acid oxidation in liver occurs in the mitochondria; hence we have examined the effects of metabolite VI on the mitochondrial oxidation of fatty acyl-*L*-carnitines. Figure 6 clearly demonstrates a concentration-related inhibition of octanoyl-*L*-carnitine metabolism by metabolite VI. Conversely, palmitoyl-*L*-carnitine oxidation was unaffected. Further studies have demon-

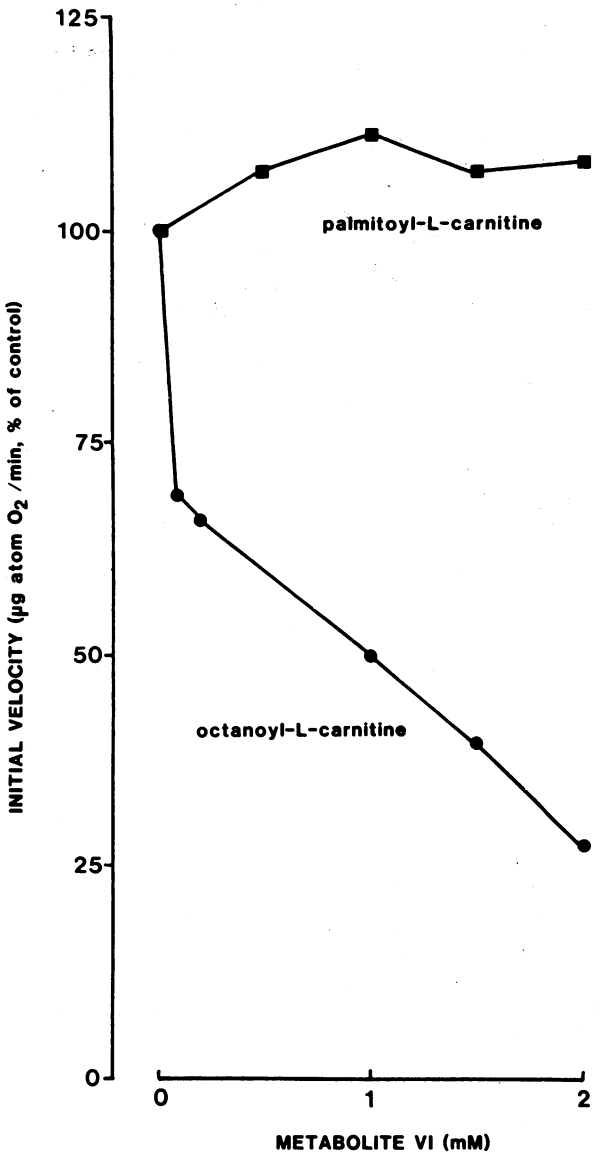

FIGURE 6. Inhibition of acylcarnitine oxidation by metabolite VI in isolated, uncoupled rat liver mitochondria. Palmitoyl-*L*-carnitine and octanoyl-*L*-carnitine were used at concentrations of 22 and 80 μM, respectively. Mitochondrial protein concentration was 1–1.5 mg/mL.

strated the inhibition of octanoyl-*L*-carnitine by metabolite VI to be competitive (data not shown).

Discussion

The *in vitro* exposure of rat hepatocytes to MEHP resulted in a marked proliferation of peroxisomes and concomitant increases in peroxisomal β-oxidation. The extent of this stimulation was similar to or greater than that observed in previous *in vivo* studies (*4,54–56*). In contradistinction to the observations made with rat hepatocytes, MEHP did not produce significant peroxisome proliferation in cultured guinea pig, marmoset or human hepatocytes. These data compare favorably to previously reported *in vivo* species differences (*4,5*).

Such differences between species could be explained by a divergence in biotransformation. For example, the guinea pig only produces small quantities of oxidized DEHP metabolites (*42*). For this reason we have attempted to exclude such metabolic factors by studying the effects of DEHP metabolites on peroxisomal enzyme activity. Using cultured rat hepatocytes we have identified metabolites VI and IX as peroxisome proliferators. In all probability, metabolite VI is the proximate proliferator, since IX is metabolized to VI, which appears to be an endpoint in the oxidation of MEHP (*53*).

The addition of metabolite VI to guinea pig, marmoset or human hepatocyte cultures resulted in little, if any, increase in peroxisomes or related enzyme activities (PCO or LAH). This suggests that, even when the confounding influence of species differences in absorption and biotransformation are eliminated, a species difference in peroxisome proliferation still exists.

These data support the suggestion of intrinsic species differences in the response of liver cells to peroxisome proliferators. However, one cannot be sure that this is not merely a quantitative difference masquerading as a qualitative difference in response. Despite this problem of semantics, it is apparent that an impressive species difference in sensitivity, if not an absolute difference in susceptibility, exists for DEHP-elicited peroxisome proliferation.

To resolve the question of quantitative or qualitative differences in response, studies aimed at elucidating the mechanism(s) of peroxisome proliferation are required. It is manifest that peroxisome proliferators elicit a diverse number of effects upon enzymes, organelles and cofactors involved in lipid metabolism. For example, increased ω- and β-oxidation of fatty acids, acyl CoA hydrolases, CoA, and carnitine are among the most common observations following administration of peroxisome proliferators to rodents (*9,12,57–61*). Furthermore, similar changes are effected by high fat diets where peroxisomal β-oxidation may be increased by up to 8-fold (*62*).

In light of these observations, we suggest that increased intrahepatic lipid may be an important factor in the genesis of peroxisome proliferation. Such an accumulation of lipid may be produced in several ways; however the present studies have shown that metabolite VI, the active proliferator derived from DEHP, inhibits fatty acid oxidation in isolated cells and selectively inhibits medium chain fatty acid oxidation in isolated mitochondria. This could lead to an accumulation of medium-chain fatty acids as their CoA or carnitine esters; hence, due to depletion of essential cofactors, leading to inhibition of all fatty acid oxidation. This would explain the inhibition of palmitic acid metabolism in isolated hepatocytes and the lack of inhibition of palmitoyl-*L*-carnitine oxidation in mitochondria by metabolite VI. Thus, one could envisage the inhibition of β-oxidation leading to accumulation of lipids and these in turn leading to an increased synthesis of organelles and

enzymes involved in fatty acid oxidation, in an attempt to maintain cellular homeostasis. Preliminary observations (Elcombe and Mitchell, unpublished) support such an accumulation of lipid, and attempts are in progress to isolate the lipids involved and examine their potencies as peroxisome proliferators. Further experiments are required to determine if this biochemical lesion of inhibition of β-oxidation is operative in species nonresponsive to peroxisome proliferators, or whether the initial lesion occurs but the cells do not respond to the accumulation of lipid.

In conclusion, our data suggest that primary cultures of hepatocytes are a sensitive and valuable model for the study of peroxisome proliferation; allowing comparative potency evaluations, the identification of proximate peroxisome proliferators, and the study of species differences. Furthermore, these systems allow the exclusion of differences in biotransformation which bedevil many *in vivo* studies utilizing the parent compound or "pro-proliferator."

From these data, we propose that an intrinsic difference in response exists between rat and human liver cells. Hence, we suggest that, for compounds such as DEHP, a rational human hazard assessment cannot be made on the basis of rat data alone. Future hazard assessments will be more predictable and accurate when a sound mechanistic basis for the phenomenon of peroxisome proliferation and its association with cancer are elucidated.

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